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(54) Title: METHOD FOR ENHANCING TRANSMEMBRANE TRANSPORT OF EXOGENOUS MOLECULES

(57) Abstract

A method is provided for enhancing transmembrane transport of exogenous molecules. A complex between a water soluble vitamin and an exogenous molecule is formed and contacted with the cell membrane thereby initiating receptor mediated transmembrane transport of the vitamin complex. The transmembrane transport of exogenous molecules including proteins and polynucleotides, as vitamin complexes, has been promoted in plant, mammalian, and bacterial cells.

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METHOD FOR ENHANCING TRANSMEMBRANE
TRANSPORT OF EXOGENOUS MOLECULES

FIELD OF INVENTION

5 This invention relates to a method for enhancing transmembrane transport of exogenous molecules. The method takes advantage of (1) the multiplicity of location and receptors in the membrane surfaces of most cells and (2) the associated receptor
10 mediated transmembrane processes. A complex between a water soluble vitamin and an exogenous molecule is formed and contacted with the membrane surface thereby initiating receptor mediated transmembrane transport of the vitamin complex. The transmembrane transport of
15 exogenous molecules including proteins and polynucleotides, as vitamin complexes, has been promoted in plant, mammalian, and bacterial cells.

BACKGROUND AND SUMMARY OF THE INVENTION

20 Transmembrane transport of exogenous or nutrient molecules is critical for normal cell function. Because practitioners have recognized the importance of that fundamental cellular process to many areas of medical and biological science, including drug
25 therapy and DNA transfection, there has been significant research efforts directed to the understanding and application of such processes. Transmembrane delivery of specific molecules has been encouraged through the use of protein carriers, antibody carriers, liposomal
30 delivery systems, electroporation, direct injection,

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cell fusion, viral carriers, osmotic shock, and calcium-phosphate mediated transfection. However, many of those techniques are limited in both the types of cells and the conditions of use for successful 5 transmembrane transport of exogenous molecular species. Further, many of these known techniques are limited in the type and size of exogenous molecule that can be transported across a membrane without loss of bioactivity.

10 One method for transmembrane delivery of exogenous molecules having a wide applicability is based on the mechanism of receptor mediated endocytotic activity. Unlike many other methods, receptor mediated endocytotic activity can be used successfully both in 15 vivo and in vitro. Receptor mediated endocytosis involves the movement of ligands bound to membrane receptors into the interior of an area bounded by the membrane through invagination of the membrane. The process is initiated or activated by the binding of a 20 receptor specific ligand to the receptor. Many receptor mediated endocytotic systems have been characterized, including galactose, mannose, mannose 6-phosphate, 25 transferrin, asialoglycoprotein, transcobolamin (vitamin B-12), α -2 macroglobulins, insulin, and other peptide growth factors such as epidermal growth factor (EGF).

Receptor mediated endocytotic activity has been utilized for transmembrane delivery of exogenous molecules such as proteins and nucleic acids. Generally, the ligand is chemically conjugated by 30 covalent, ionic or hydrogen bonding to exogenous

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molecule of interest, (i.e., the exogenous compound) forming a conjugate molecule having a moiety (the ligand portion) that is still recognized in the conjugate by a target receptor. Using this technique the phototoxic 5 protein psoralen has been conjugated to insulin and internalized by the insulin receptor endocytotic pathway (Gasparro, Biochem. Biophys. Res. Comm. 141(2), pp. 502-509, Dec. 15, 1986); the hepatocytes specific receptor for galactose terminal asialo-glycoproteins has 10 been utilized for the hepatocytes-specific transmembrane delivery of asialoorosomucoid-poly-L-lysine non-covalently complexed to a DNA plasmid (Wu, G.Y., J. Biol. Chem., 262(10), pp. 4429-4432, 1987); and the cell receptor for epidermal growth factor has been utilized 15 to deliver polynucleotides covalently linked to EGF to the cell interior (Myers, European Patent Application 86810614.7, Filed December 29, 1986, Publication Date June 6, 88).

The method of the present invention enhances 20 the transmembrane transport of an exogenous molecule across a membrane having receptors for water soluble vitamins that initiate transmembrane transport following binding with a water soluble vitamin or a pharmacologic agent that mimics the binding of a water soluble 25 vitamin. The present method has been successfully applied to mammalian, plant, and bacterial cells. The vitamin receptor mediated transmembrane transport forming the basis of this invention is initiated by the binding of water soluble vitamins, such as biotin, 30 ascorbic acid, cobalamine, or folates, to their

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respective receptor associated with the membrane. A preferred target receptor for the method of the present invention is the biotin receptor. Biotin is a necessary cellular growth factor that has been found to be 5 preferentially bound by biotin receptor proteins associated with cellular membranes. Biotinylating reagents suitable for covalently bonding a biotin moiety to polynucleotides, proteins, or other desired molecules are commercially available. The binding of the vitamin 10 moiety to its cell surface receptor initiates transmembrane transport of the water soluble vitamin or, in the case of the present invention, the complex consisting of the vitamin and the exogenous molecule.

The present invention makes use of a 15 receptor-mediated transmembrane transport to deliver exogenous molecules complexed with the water soluble vitamin, across a membrane. A complex is first formed between a water soluble vitamin and a predetermined exogenous molecule. The complex is then contacted with 20 a cell having receptors for water soluble vitamins and an associated receptor mediated transmembrane transport activity for a time sufficient to permit transmembrane transport of the complex by water soluble vitamin receptor mediated transmembrane transport activity. In 25 this manner, exogenous molecules are either transported, or transported at an enhanced rate, across the membrane.

The method of the present invention is 30 particularly useful in increasing the internalization yields (cellular uptake) of exogenous molecules that normally are resistant to cellular internalization.

Proteins and polynucleotides previously recognized as difficult to move across cell membranes can be internalized by the method of the present invention. For example, transfection and expression of an encoded 5 protein product by an internalized biotin-complexed functional gene has been demonstrated. Biotin, conjugated with a DNA plasmid containing a gene sequence coding for chloramphenicol acetyltransferase (CAT), was transported into E. coli via a biotin receptor mediated 10 endocytotic pathway and expressed. Transport of biotinylated protein products into both mammalian and plant cells has also been achieved in both *in vivo* and *in vitro* systems.

The method of the present invention can also be 15 accomplished utilizing chemical analogues or derivatives of water soluble vitamins that are cross reactive with a water-soluble-vitamin receptor.

DETAILED DESCRIPTION OF THE INVENTION

20 The method of the present invention requires the presence of appropriate receptors for water soluble vitamins associated with a membrane. The membrane can either define an intracellular volume such as the endoplasmic reticulum or other organelles such as 25 mitochondria, or alternatively can define the boundary of the cell. Transmembrane transport across a cell boundary commonly occurs by an endocytotic transport mechanism. Generally, it has been found that water soluble vitamin receptors mediate cellular 30 internalization of water soluble vitamins through

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endocytotic activity. The receptors can be natural constituents of the cell or they can be emplaced in the cell membrane by external physical manipulation.

Alternatively, expression of an inserted foreign gene 5 for the protein or apoprotein corresponding to the water soluble vitamin receptor by a transfected cell can ensure the presence of a water soluble vitamin receptor on a target cell.

Water soluble vitamins known or believed to 10 have suitable cellular receptors for purposes of the present invention include but are not limited to biotin, biotin analogues such as 6-N-biotinyl-L-lysine (biocytin), biotin sulfoxide, oxybiotin (oxobiotin), 5,6,-dimethylbenzimidazoloylcyanocobamide 15 (cyanocobalamin - vitamin B-12), 5,6,-dimethylbenzimidazoloylaquaocobamide (aquocobalamin - vitamin B-12_a), 5,6,-dimethylbenzimidazoloylhydroxocobamide (hydroxocobalamin - vitamin B-12_b), adenosylcobalamin, 20 methylcobalamin, folic acids such as folacin, methotrexate, pteropolyglutamic acid, pteridines, niacin, pantothenic acid, riboflavin, and thiamin.

Preliminary experiments using the water soluble 25 vitamin pyridoxine showed little uptake potentiating activity. It is possible that pyridoxine and pyridoxine analogues are not suitable for use in accordance with the present invention.

Because of the ready availability of 30 biotinylation reagents and biotinylation methods suitable for use with peptides, proteins,

oligonucleotides, and polynucleotides, a preferred water soluble vitamin for the purposes of the present invention is biotin. Biotin is also a preferred water soluble vitamin because it is a necessary growth factor for a wide variety of cells, and biotin receptors that mediate endocytotic activity have been identified in mammalian, plant, and bacterial cells.

Formation of a complex between a water soluble vitamin such as biotin and an exogenous molecule of interest is readily accomplished for a great many molecules and macromolecules. Biotin moieties can be easily conjugated to proteins by making the carboxyl group of biotin reactive toward the free amino groups of the proteins. A biotinylating reagent such as D-biotin-N-hydroxy-succinimide ester or biotinyl-p-nitrophenyl ester can be used. The activated ester reacts under mild conditions with amino groups to incorporate a biotin residue into the desired molecule. The procedure to be followed for biotinylating macromolecules using D-biotin-N-hydroxy-succinimide ester is well known in the art (Hofmann et al., J.Am.Chem.Soc. 100, 3585-3590 (1978)). Procedures suitable for biotinylating an exogenous molecule using biotinyl-p-nitrophenyl ester as a biotinylating reagent are also well known in the art (Bodanszk et al., J.Am.Chem.Soc. 99, 235 (1977)). Other reagents such as D-biotinyl- ϵ -aminocaproic acid N-hydroxy-succinimide ester in which ϵ -aminocaproic acid serves as a spacer link to reduce steric hindrance can also be used for the purposes of the present invention.

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Oligonucleotides and polynucleotides can also be biotinylated using both indirect and direct methods. Indirect methods include end-labeling of a polynucleotide with a biotinylated nucleotide, or nick 5 translation that incorporates biotinylated nucleotides. Nick translation or end labeling of DNA can be accomplished using methods described in Maniatis et al., Molecular Cloning: A Laboratory Manual, pp. 109-116, Cold Spring Harbor Press (1982).

10 Direct methods refer to those procedures in which biotin is directly attached to a target polynucleotide using a biotinylating reagent. Photoactivatable reagents such as the acetate salt of N-(4-azido-2-nitrophenyl)-N-(3-biotinylaminopropyl)-N-15 methyl-1,3-propanediamine (photobiotin) can be used to biotinylate DNA according to the method of Forster et al., Nuc. Acids Res. 13:745-761. An alternative method uses a biotin hydrazide reagent in a bisulfite catalyzed reaction capable of transamination of nucleotide bases 20 such as cytidine according to the method described by Reisfeld et al., B.B.R.C. 142:519-526 (1988). This method simply requires a 24 hour incubation of DNA or RNA with biotin hydrazide at 10mg/ml in an acetate buffer, pH 4.5, containing 1 M bisulfite. Biotin 25 hydrazide can also be used to biotinylate carbohydrates containing a free aldehyde.

30 Water soluble vitamin receptor mediated cellular uptake of biotinylated polynucleotides provides an alternative mechanism for transfection of cells. The technique of the present invention is particularly

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valuable because it is applicable to certain cell types, such as plant cells, which are normally resistant to standard transfection techniques. Delivery of foreign genes to the cell interior can be enabled or enhanced by 5 the present invention. Once delivered to the cellular interior, these foreign genes can be inserted and expressed with the aid of a natural or exogenous promoter to produce a desired protein. In addition to proteins, other useful macromolecules can be produced. 10 For example, an antisense-RNA sequence capable of binding interference with endogeneous messenger RNA.

The delivery of proteins and other non-nucleotide molecules by water soluble vitamin receptor mediated uptake is also useful. Antibodies, 15 bioactive peptides, toxic peptides, or pharmaceutically valuable peptides can be delivered to the cellular interior by means of the present invention. This is of particular value for in vivo, therapeutic applications involving the delivery of molecules that are not 20 normally internalized by a target cell.

The following examples are provided to illustrate further the range of exogenous molecules and cell types to which the method of the present invention can be applied.

25 Example 1 - RAT PHEOCHROMOCYTOMA CELL UPTAKE OF BIOTIN CONJUGATED INSULIN:

Rat pheochromocytoma (PC-12) cells were obtained from America Type Culture Collection and were grown (37°C, 5% CO₂ in humidified air) attached to 30 plastic flasks for 2 to 3 weeks until confluent in a

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medium of 85% RMPI 1640, 10% v/v heat inactivated horse serum, and 5% fetal calf serum containing 1% streptomycin-penicillin.

5 Biotin and fluorescein labeled insulin was prepared. To 1 ml of a 1 mg/ml solution of insulin protein in phosphate buffered saline was added simultaneously 100 μ l of a 1 mg/ml solution of fluorescein isothiocyanate (FITC) in dimethylformamide (DMF) and 100 μ l of a 1 mg/ml solution of 10 N-hydroxysuccinimido biotin in dimethylsulfoxide (DMSO). The two labeling reagents were allowed to react at room temperature for 4 hours, after which the unreacted reagents were quenched with 10 μ l ethanolamine. The quenched reaction mixture was then 15 dialyzed against double distilled water until unreacted fluorescein derivatives no longer dialyzed into the water. The covalent attachment of biotin and fluorescein to the desired protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel 20 electrophoresis and western blot analysis.

As a control, non-biotinylated fluorescein labeled insulin was prepared. 1 ml of a 1 mg/ml solution of insulin was added 0.5 ml of a 1 mg/ml solution of fluorescein isothiocyanate (FITC) in 25 dimethylformamide (DMF). The reaction was allowed to proceed for 4 hours in the dark at room temperature. After 4 hours the reaction was quenched with 10 μ l ethanolamine, and the labeled insulin solution was dialyzed against double distilled water until unreacted 30 FITC no longer appeared in the solution.

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The rat PC12 cells were grown in modified RMPI 1640 medium as a monolayer on the bottom of a culture flask. Before removing the cells, the monolayer was washed with a 20 ml portion of fresh Locke's solution.

5 The cells were then displaced into 20 ml of the Locke's solution by gentle agitation with a stream Locke's solution. The suspended cells were pelleted by centrifugation at 10,000 x g for 10 seconds and after resuspending in Locke's solution in separate

10 polycarbonate tubes (40ml/tube) to a final density of 1.14×10^6 cells/ml, the following amounts of proteins were added to the cell suspensions: 40 μ g fluorescein-labeled insulin was added to the first tube, and to the control tube was added 40 μ g

15 biotin-conjugated insulin labelled with fluorescein. The tubes were allowed to incubate at 37°C. At intervals of 5, 15 and 33 minutes, 0.5 ml of each cell suspension was removed and pelleted at 10,000 x g for 10 seconds. The cell pellet was washed and repelleted

20 twice in 1 ml Locke's solution and then fixed by addition of 200 μ l of a 2% formalin solution in phosphate buffered saline. Thirteen microliters of the fixed cell suspension was then added to a microscope slide and viewed with the fluorescent microscope to

25 detect internalized proteins. No evidence of internalization was noted for the fluorescein labelled insulin acting as a control. Cellular internalization was indicated for the biotinylated insulin labelled with fluorescein, with the amount internalized increasing

30 with time.

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Example 2 - RAT PHEOCHROMOCYTOMA CELL UPTAKE OF BIOTIN CONJUGATED HEMOGLOBIN:

Following the same general procedure set forth in Example 1 hemoglobin was biotinylated, and the biotinylated form was shown to be preferentially internalized by rat pheochromocytoma cells as compared to non-biotinylated hemoglobin.

Example 3 - SOYBEAN CELL UPTAKE OF BOVINE SERUM ALBUMIN:

Soybean cell suspension cultures of Glycine max Merr Var Kent were maintained by transferring cells to fresh W-38 growth medium every 7 days.

To 20 ml of a suspension culture of soybean cells was added 10 μ g of either fluorescein-labeled (control) or fluorescein and biotin labelled bovine serum albumin. The cells were allowed to incubate for up to 6 hours. At varying time intervals 1 ml of the cell suspension was filtered to remove the growth medium, washed with 50 ml fresh growth medium, and resuspended in 20 ml of the same medium. The cell suspension was then viewed with a fluorescent microscope to determine whether cellular internalization of the labelled bovine serum albumin had occurred. Cellular internalization was indicated only for biotinylated bovine serum albumin.

Example 4 - SOYBEAN CELL UPTAKE OF INSULIN:

Following the same general procedure set forth in Example 3 insulin was biotinylated, and the biotinylated form of insulin was shown to be preferentially internalized by soybean cells as compared to non-biotinylated insulin.

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Example 5 - SOYBEAN CELL UPTAKE OF HEMOGLOBIN:

Following the same general procedure set forth in Example 3 hemoglobin was biotinylated, and the biotinylated form of hemoglobin was shown to be 5 preferentially internalized by soybean cells as compared to non-biotinylated hemoglobin.

Example 6 - CARROT CELL UPTAKE OF BOVINE SERUM ALBUMIN:

Carrot cells of wild type origin were established and maintained in MS growth medium 10 supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid. Bovine serum albumin was labelled with fluorescein alone as a control or with fluorescein and biotin following the procedures detailed in Example 3. The carrot cells were then incubated in the presence of 15 the respective labelled bovine serum albumin for 7 hours. All other conditions were the same as those described in Example 3 above. Cellular internalization was found only in those cells contacted with biotin labelled bovine serum albumin.

Example 7 - CARROT CELL UPTAKE OF INSULIN:

Following the same general procedure set forth in Example 6 insulin was biotinylated, and the biotinylated form was shown to be preferentially 25 internalized by carrot cells as compared to non-biotinylated insulin.

Example 8 - CARROT CELL UPTAKE OF HEMOGLOBIN:

Following the same general procedure set forth in Example 6 hemoglobin was biotinylated, and the biotinylated form was shown to be preferentially 30 internalized by carrot cells as compared to non-biotinylated hemoglobin.

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Example 9 - SOYBEAN CELL DEGRADATION OF HEMOGLOBIN:

To determine whether hemoglobin was rapidly degraded following cellular internalization by transmembrane transport, soybean cells were allowed to 5 internalize and metabolize biotinylated hemoglobin for a period of 8 hours under conditions described in Example 5, after which the soybean cells were rapidly homogenized in a sodium dodecyl sulfate solution to 10 disaggregate and denature all protein material. The solubilized polypeptides were separated according to 15 molecular weight by polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose paper. The positions of the biotin-labeled peptides were then visualized on the nitrocellulose blot by staining with horseradish peroxidase-linked avidin and the colored 20 substrate, p-chloronaphthol. All of the biotin-linked material was found to migrate with an apparent molecular weight of ~16,000 daltons, about equal to the molecular weight of the parent globin chains of 25 hemoglobin, indicating no breakdown of the parent globin chains had occurred during the 8 hour incubation period.

Example 10 - IN VIVO DELIVERY TO RATS OF SOYBEAN TRYPSIN INHIBITOR:

Soybean trypsin inhibitor (SBTI) (-6 mg) was 25 labeled with radioactive ¹²⁵I using 8 iodobeads (Bio Rad) in 1 mL buffer which was then dialyzed to remove unreacted ¹²⁵I. After dividing into two equal fractions, one fraction was biotinylated with 30 N-hydroxysuccinimidyl biotin and the other fraction was left as an unmodified control. Mice (~25 g) were then

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injected with either the biotinylated SBTI or the control SBTI by insertion of a hypodermic syringe containing a 25 gauge needle into the tail vein of the mouse. After 15 minutes, each mouse was sacrificed and then perfused with heparin-containing isotonic saline via the direct cardiac influx and efflux method. When the various tissues appeared to be blood-free, the perfusion was terminated and each tissue/organ was removed, weighed, and counted for ^{125}I -SBTI in a gamma counter. Although some radioactivity was detected in the mice treated with non-biotinylated ^{125}I -SBTI, between 4 and 100 times more ^{125}I -SBTI was found in the mice treated with biotinylated SBTI, indicating successful in vivo delivery to murine cellular tissue.

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Counts per minute/gram wet weight

	<u>Tissue</u>	<u>Control SBTI</u>	<u>Biotin SBTI</u>
20	Liver	535	1967
	Lung	107	2941
	Kidney	5152	8697
	Intestine	0	700
	Muscle	0	1065
25	Heart	0	739
	Brain	0	267

Example 11 - SOYBEAN TRANSFECTION OF SALMON SPERM DNA:
Protein free salmon-sperm DNA, either in a
30 highly polymerized form (\geq 50,000 base pair length) or

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in a sheared form (\leq 500 base pair length), was transaminated at the cytosine residues. The transaminated DNA (1 mg) was labeled with fluorescein via the addition of 0.5 mg of fluorescein isothiocyanate (FITC) in dimethylsulfoxide (DMSO). The resulting reaction mixture was divided into two portions and the labeling reaction was quenched in one portion by addition of 10 μ L of ethanolamine. This quenched portion served as the non-biotinylated control. The remaining DNA was then covalently labeled with biotin via reaction with 0.5 mg of N-hydroxysuccinimidyl biotin in DMSO. After purification, the two derivatives (1 μ g/ml) were separately incubated with soybean suspension culture cells at room temperature for 6 hours and then the cells were washed with 50 ml fresh growth medium and observed by fluorescence microscopy. Only the biotinylated DNA entered the soybean cells.

Example 12 - E. COLI TRANSFECTION AND EXPRESSION OF AMPICILLIN RESISTANT GENE:

Plasmid DNA (pUC8) was biotinylated via nick translation in the presence of biotin-14-dATP using a commercially available nick translation kit (Bethesda Research Laboratories). The biotinylated DNA and unmodified DNA (1 μ g) were added to E. coli strain C_u 1230 that had been made competent by treatment with MgCl₂ and CaCl₂ following the method of Maniatis et al., Molecular Cloning: A Laboratory Manual, pp. 250-251, Cold Spring Harbor Press (1987). After transformation, the successful transformants were selected by plating cells on LB media which contained 50

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µg/ml ampicillin and then incubated overnight at 37°C. Colonies which survived the ampicillin were counted and the transformation efficiency was determined. The number of surviving E. coli colonies 5 was at least 100-fold greater in E. Coli transformed with the biotinylated plasmids.

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WHAT IS CLAIMED IS:

1. A method for enhancing transport of an exogenous molecule into a plant cell, said method comprising the step of contacting the plant cell with the exogenous molecule complexed with a water soluble vitamin or water soluble vitamin receptor binding agent for a time sufficient to permit transmembrane transport of said complex.
- 10 2. The method of claim 1 wherein the water soluble vitamin is biotin or analogs thereof.
3. The method of claim 1 or 2 wherein the exogenous molecule is a nucleic acid.
- 15 4. The method of claim 1 or 2 wherein the exogenous molecule is a protein.
5. A method for enhancing transport of a nucleic acid into a cell, said method comprising the step of contacting the cell with the nucleic acid complexed with a water soluble vitamin or water soluble vitamin receptor binding agent for a time sufficient to permit transmembrane transport of said complex.
- 20 6. The method of claim 5 wherein the water soluble vitamin is biotin or analogs thereof.
7. The method of claim 5 or 6 wherein the cell is an animal cell.
- 25 8. The method of claim 5 or 6 wherein the cell is a plant cell.
9. The method of claim 5 or 6 wherein the cell is a prokaryote.
- 30 10. A method for enhancing transport of an exogenous molecule into cellular tissue of a host having

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a circulatory system, said method comprising the step of injecting the host with the exogenous molecule complexed with a water soluble vitamin or water soluble vitamin receptor binding agent.

5 11. The method of claim 10 wherein the water soluble vitamin is biotin or analogs thereof.

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01722

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00

U.S. CL.: 435/6.7

II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched ¹	
		Classification Symbols
U.S. CL.	435/6.7, 172.3,	
	536/27	935/56, 58, 52

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ²

APS: biotin, introduc., cell(s)

III. DOCUMENTS CONSIDERED TO BE RELEVANT ^{1a}

Category ³	Citation of Document, ^{1b} with indication, where appropriate, of the relevant passages ^{1c}	Relevant to Claim No. ^{1d}
Y	US, A, 4,683,205 issued 28 July 1987, (KATSUMATA, ET AL.), see entire document.	1-11
Y	Methods of Biochemical Analysis, volume 26 issued 1978, (BAYER ET AL.), "The use of the avidin-biotin complex as a tool in molecular biology", see pages 1-45.	1-11
Y	Biochemistry et Biophysic Acts Volume 721 issued 1982 (CHALIFOUR ET AL.), "The characterization of the uptake of avidin-biotin complex by Hela Cells", see pages 64-69.	1-11
Y	Annual Review of Physiology, Volume 43 issued 1981, (PASTAN ET AL.), "Receptor-mediated endocytosis of hormones in cultured cells", See pages 239-250.	1-11

* Special categories of cited documents: ^{1e}

- "A" document defining the general state of the art which is not considered to be of particular relevance
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search ²:

22 JUNE 1990

Date of Mailing of this International Search Report ²:

01 AUG 1990

International Searching Authority ²:

ISA/US

Signature of Authorized Officer ^{2a}

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